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13. ABSTRACT *(Maximum 200 Words)*
CpG island hypermethylation is a frequent epigenetic event in cancer. We have recently developed an array-based method, called differential methylation hybridization (DMH), allowing for a genome-wide screening of CpG island hypermethylation in breast cancer cell lines (Hum. Mol. Genet. *8:* 459-470, 1999). In the present study, DMH was applied to screen 28 paired primary breast tumor and normal samples, and to determine whether patterns of specific epigenetic alterations correlate with pathological parameters in the patients analyzed. Amplicons, representing a pool of methylated CpG DNA derived from these samples, were used as hybridization probes in an array panel containing 1,104 CpG island tags. Close to 9% of these tags exhibited extensive hypermethylation in the majority of breast tumors relative to their normal controls, while others had little or no detectable changes. Pattern analysis in a subset of CpG island tags revealed that CpG island hypermethylation is associated with histological grades of breast tumors. Poorly differentiated tumors appeared to exhibit more hypermethylated CpG islands than their moderately or well-differentiated counterparts (P=0.041). This early finding lays the groundwork for population-based DMH study and demonstrates the need to develop a database for examining large-scale methylation data and for associating specific epigenetic signatures with clinical parameters in breast cancer.

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(5) Introduction

Cancer is a complex disease, resulting from multiple genetic mutations of genes. One less known phenomenon is "epi"genetic mutations also frequently observed in cancer. This epigenetic mutation occurs by converting cytosine bases (Cs) to methylated Cs (mCs) via a chemical reaction, called DNA methylation (1). As a result of this epigenetic imbalance, gene regulation specific for a cell type is disrupted, leading to tumor formation (2). During this granting period, we have applied differential methylation hybridization (DMH), a high-throughput microarray-based technique, for simultaneous analysis of methylated Cs across thousands of GC-rich region genes in primary breast cancer specimens (3). Our approach can generate a vast amount of information for unique molecular identifiers, i.e., epigenetic signatures, of individual cancers. This type of study will pave the way for complementing the histopathological examinations currently in use with molecular diagnosis and classification of tumors in the future.

(6) Body

a) Develop differential methylation hybridization (DMH) technique (corresponding to Task 1). The development of DMH technique has previously been described in detail in the last progress report. During this granting period, we have continued to expand our repertoire of CpG island clones. As of August 2000, a total of 22,000 CpG island tags have been established in our laboratory. These tags have been generated by PCR and have been and will be printed on nylon membranes or on glass slides for genome-wide methylation analysis in breast tumors as well as other types of cancer.

b) Determine the effects of abnormal DNA methylation in breast cancer cells (corresponding to Task 2). In this study, we determined whether DMH alone could be routinely applied to identify CpG island hypermethylation in clinical specimens. We analyzed 28 paired normal and breast tumor specimens with an array panel representing about 2% of total CpG islands in the genome. DMH was initially applied to 28 paired breast tumor and normal samples using an array panel containing >1,000 CpG island tags. Fig.1 shows representative results of DMH screening in paired samples of patients 121, 103, and 91 (see Yan *et al.*, Clin. Cancer Res. in Appendix). Based on visual survey, hypermethylated sequences were identified in breast tumors, showing detectable hybridization signals in CpG island tags probed with tumor amplicons, but not in the same tags probed with normal amplicons. This is because methylated *Bst*UI sites in tumor DNA were protected from restriction within CpG island sequences, which were then amplified by linker-PCR and hybridized to the corresponding tags. The same sites, however, were unmethylated or partially methylated in normal DNA and were restricted by *Bst*UI; therefore, no hybridization signals were detected in the arrays.

Methylation data derived from DMH were correlated with pathological parameters in the 28 patients analyzed. Statistical analysis revealed that CpG island hypermethylation was associated with histological grades of breast tumors (P=0.041). To aid us in visualizing differences in CpG island hypermethylation among different tumor grades, we devised a gray scale by categorizing tumor methylation volumes into percentiles as depicted in Fig. 3 (see Yan *et al.*, Clin. Cancer Res. in Appendix). The poorly differentiated (PD) group exhibited more frequent and extensive hypermethylation at the loci tested than their moderately differentiated (MD) and well-differentiated (WD) counterparts did; half of the 14 PD tumors showed extensive hypermethylation at multiple loci (>10), while only two of the 14 MD/WD tumors showed hypermethylation at these loci. Moreover, the greatest degrees of differences were seen at loci HBC-42, -45, and -47 that were frequently hypermethylated in PD tumors, but not in MD/WD. This result suggests that patients with more advanced disease status are prone to methylation alterations. It should be noted that some of the patients showed little or no changes of methylation at the loci tested. This indicates that progression of some tumors may be independent of this epigenetic event or the alteration could occur in later stages of tumor development in such patients. No association of hypermethylation with other clinical parameters was found in this study.

c) Characterization of hypermethylated CpG island loci by nucleotide sequencing (corresponding to Task 3). Thirty CpG island tags positive for hypermethylation in the primary screening were selected for further characterization. DNA sequencing results showed that 9 of these tags contained sequences identical to known

cDNAs, *PAX7* (5' end), *Caveolin-1* (exon 2), *GATA-3* (exon 1), *MAR M1H12* (AA604922), and *COL9A1* (exon 1), and 4 ESTs (AI381934, AA315965, AI500696, and F211078) (for sequencing data, see http://www.missouri.edu/~hypermet). Twenty-five tags were further numerically assigned as HBC (Hypermethylation in Breast Cancer)-33 to -57 following a previous series of studies (3). This finding is consistent with that of Engelman et al. (4), where they also observed CpG island methylation in the *Caveolin*-1 gene in breast cancer cell lines. Five other tags, HBC-17, 19, 24, 25, and 27, found to be hypermethylated in breast cancer cell lines as reported previously, were also identified in this study. (As an ongoing process, we will continue to characterize positive CpG island sequences identified using DMH in Task 2.)

(7) Key Research Accomplishments

- A panel of 22,000 CpG island tags has been established for genome-wide methylation analysis using DMH.
- Profiles of epigenetic alterations in tumors can potentially be used for cancer diagnosis and classification
- Poorly differentiated tumors appeared to exhibit more hypermethylation than moderately or well-differentiated tumors (P=0.041).

(8) Reportable Outcomes

- One manuscript was published in Clinical Cancer Research. The same manuscript was presented in the 2000 Era of Hope meeting in Atlanta. Additional papers were published in British Journal of Cancer and Breast Cancer Research and Treatment (see the Appendix).

(9) Conclusion

In conclusion, our results demonstrate the power of DMH with hybridization arrays to rapidly generate information on patterns of differentially methylated CpG islands that may then be moved on to population-based studies of the epigenotype-phenotype relationship.

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CpG Island Arrays: An Application toward Deciphering Epigenetic Signatures of Breast Cancer[1]

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ABSTRACT

CpG island hypermethylation is a frequent epigenetic event in cancer. We have recently developed an array-based method, called differential methylation hybridization (DMH), allowing for a genome-wide screening of CpG island hypermethylation in breast cancer cell lines (T. H-M. Huang *et al.*, Hum. Mol. Genet., *8:* 459–470, 1999). In the present study, DMH was applied to screen 28 paired primary breast tumor and normal samples and to determine whether patterns of specific epigenetic alterations correlate with pathological parameters in the patients analyzed. Amplicons, representing a pool of methylated CpG DNA derived from these samples, were used as hybridization probes in an array panel containing 1104 CpG island tags. Close to 9% of these tags exhibited extensive hypermethylation in the majority of breast tumors relative to their normal controls, whereas others had little or no detectable changes. Pattern analysis in a subset of CpG island tags revealed that CpG island hypermethylation is associated with histological grades of breast tumors. Poorly differentiated tumors appeared to exhibit more hypermethylated CpG islands than their moderately or well-differentiated counterparts ($P = 0.041$). This early finding lays the groundwork for a population-based DMH study and demonstrates the need to develop a database for examining large-scale methylation data and for associating specific epigenetic signatures with clinical parameters in breast cancer.

INTRODUCTION

The recent development of high-throughput technologies has provided powerful tools for comprehensive genome-wide analysis of gene expression, chromosomal alterations, and nucleotide mutations in cancer. These technologies, including oligonucleotide (1), cDNA (2, 3), and tissue (4) microarrays and serial analysis of gene expression (5), can generate a vast amount of molecular information and may provide comprehensive profiles of genetic changes for different types of cancer cells. Translational research is just beginning to search unique molecular identifiers, *i.e.,* genetic signatures, in clinical tumor specimens. This type of study will pave the way for complementing the histopathological examinations currently in use with molecular tumor diagnosis and classification in the future.

Until recently, such high-throughput technological development has been lacking for another common molecular alteration, *i.e.,* DNA methylation, in the tumor genome. This epigenetic alteration usually occurs by deleting or adding a methyl group in the fifth carbon position of a cytosine located 5′ of a guanine known as CpG dinucleotide (6). Loss of cytosine methylation, or hypomethylation, is frequently observed in bulk chromatin or repetitive sequences and may promote gross chromosomal rearrangements (6–8). In contrast, *de novo* cytosine methylation, or hypermethylation, is a regional event that occurs frequently in GC-rich sequences, called CpG islands, located within the 5′ regulatory regions of nontranscribed genes (6, 9).

We have recently adapted the microarray-based strategy and developed a novel technique, called DMH,[3] for the first time, providing a tool that can efficiently scan the tumor genome for methylation alterations (10). The first part of DMH is the generation of GC-rich tags derived from a human CpG island genomic library, CGI (11). These tags were then arrayed onto solid supports (*e.g.,* nylon membranes). The second part involves the preparation of amplicons, representing a pool of methylated CpG DNA, from tumor or reference samples. Amplicons are used as probes for CpG island array hybridization. The differences in tumor and reference signal intensities on CpG island arrays tested reflect methylation alterations of corresponding sequences in the tumor DNA. DMH was successfully applied to detect specific methylation profiles in a group of breast cancer cell lines, and hypermethylation of CpG island loci was independently confirmed by Southern-based analysis (10). Subsequent pattern analysis of the positive loci revealed potential mechanisms governing aberrant methylation in these cells.

In this study, we determined whether DMH alone can be routinely applied to identify CpG island hypermethylation in

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[3] The abbreviations used are: DMH, differential methylation hybridization; CGI, CpG island; HBC, hypermethylation in breast cancer; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated.

clinical specimens. We analyzed 28 paired normal and breast tumor specimens with an array panel representing ~2% of total CpG islands in the genome. Methylation data derived from DMH were correlated with pathological parameters in the patients analyzed. Our results suggest that increased CpG island hypermethylation is associated with high-grade tumors. This initial study lays the groundwork for further population-based analysis to examine the epigenotype-phenotype relationship in breast cancer.

MATERIALS AND METHODS

Patient Samples. Breast tumor specimens were obtained from 28 female patients undergoing mastectomies at the Ellis Fischel Cancer Center (Columbia, MO) between 1992 and 1998. Adjacent, normal parenchyma was obtained from the same patient to serve as a normal control. All tumors used in this study were classified as infiltrating ductal carcinomas and were graded based on the Nottingham modified criteria of Bloom and Richardson (12). This tumor-grading method was based on histological features of tubule formation, nuclear pleomorphism, and mitotic activity, and points were assigned for each category accordingly. The overall tumor grade was the sum total of scores between 3 and 9. Tumors with poorly differentiated phenotypes (8–9 points) are likely to have fewer or no tubular structures, irregular and large nuclei, and high mitotic counts. Tumors with moderately (6–7 points) or well-differentiated (3–5 points) phenotypes may have definite tubule formation, moderate outlines of epithelial cell shapes and uniformity of nuclear chromatin, and low mitotic indexes. Patient clinical information is shown in Table 1. High molecular weight DNA was isolated from these specimens using the QIAamp tissue kit (Qiagen).

Table 1 Clinicopathological information for patients with infiltrating ductal carcinoma of the breast

Patient no.	Age at diagnosis (yr)	Clinical stage[a]	Histological grade	Estrogen receptor	Progesterone receptor
097	57	II	WD	+	−
157	49	II	WD	+	+
025	42	II	MD	−	−
045	44	III	MD	+	−
063	52	III	MD	+	+
067	40	IV	MD	−	−
071	33	II	MD	−	−
083	45	III	MD	+	+
085	64	II	MD	+	+
091	55	II	MD	−	−
095	53	I	MD	+	+
109	66	III	MD	+	+
137	82	I	MD	+	−
187	83	II	MD	+	+
031	60	II	PD	−	−
043	35	III	PD	−	−
047	38	III/IV	PD	−	−
065	42	IV	PD	−	−
089	83	III	PD	+	−
103	67	II	PD	+	−
119	67	III	PD	−	−
121	52	III	PD	−	−
123	38	II	PD	−	−
129	76	II	PD	+	+
135	46	I	PD	−	−
153	50	I	PD	−	−
155	43	—	PD	−	−
167	40	III	PD	−	−

[a] Classification according to the TNM system (19).

Amplicon Generation. DMH was performed as described previously (10). Briefly, genomic DNA (0.5–1 μg) from breast tumor or normal samples was digested with a 4-base TTAA cutter *Mse*I known to restrict DNA into small fragments (<200-bp) but leave CpG islands relatively intact (11). The cleaved ends of the digests were ligated with 0.5 nmol of linkers, H-12/H-24 (H-12, 5′-TAA TCC CTC GGA; and H-24, 5′-AGG CAA CTG TGC TAT CCG AGG GAT), in a buffer containing 400 units of T4 DNA ligase (New England Biolabs) at 16°C. After ligation, repetitive Cot-1 DNA was removed from the ligated products using a subtractive hybridization technique (13). The subtracted DNA was further digested with methylation-sensitive *Bst*UI (cuts CGCG, but not ^{m}CGCG, ^{m}C: 5-methylcyotosine). This endonuclease was used in the analysis, because >77% of known CpG islands contain *Bst*UI sites (11). Linker-PCR reactions were performed with the pretreated DNAs (~50 ng) in a 20-μl volume, containing 0.4 μM H-24 primer, 1 unit Deep Vent (exo$^-$) DNA polymerase (New England Biolabs), 5% (v/v) DMSO, and 200 μM deoxynucleotide triphosphates in a buffer provided by the supplier. The tubes were incubated for 3 min at 72°C to fill in 5′ protruding ends of ligated linkers and subjected to 15 cycles of amplification, as described previously (10). Low amplification cycles were used to prevent an overabundance of leftover repetitive sequences generated by PCR. In addition, under this condition all amplification is expected to be in the linear range of the assay, allowing for semiquantitation of dot intensities described later. The amplified products, labeled as normal or tumor amplicons, were purified and ^{32}P-labeled for array hybridization.

Array Hybridization. *Bst*UI-positive, Cot-1-negative, CpG island clones were prepared from the CGI genomic library and used for 96-well format PCR as described previously (10). PCR products (0.2–1.5 kb) were denatured and dotted in duplicate on nylon membranes using a 96-pin Multi-Print replicator (V & P Scientific). Alignment devices (Library Copier; V & P Scientific) were used in conjunction with the replicator to convert multiple 96-well PCR samples into one recipient of 576 or 1536 dots on a 10 × 12-cm nylon membrane. Membranes were first hybridized with normal amplicons, and autoradiography was conducted using the Molecular Dynamics PhosphorImager. Probes were stripped, and the same membranes or duplicate membranes were hybridized with tumor amplicons and scanned with the PhosphorImager.

Data Analysis. Dot intensities for positive CpG island tags were measured using the volume review protocol of ImageQuant software (Molecular Dynamics). The raw volume data from tumor and normal samples were normalized prior to comparison. This was achieved by ratio determination of the internal control tags. Briefly, two internal control tags with close volume ratios were selected to estimate hybridization differences between paired amplicons. One of these two control tags from each amplicon was further used to calculate a factor for normalization:

$$\text{Normalization factor} = \frac{\text{Normal internal control tag volume}}{\text{Tumor internal control tag volume}}$$

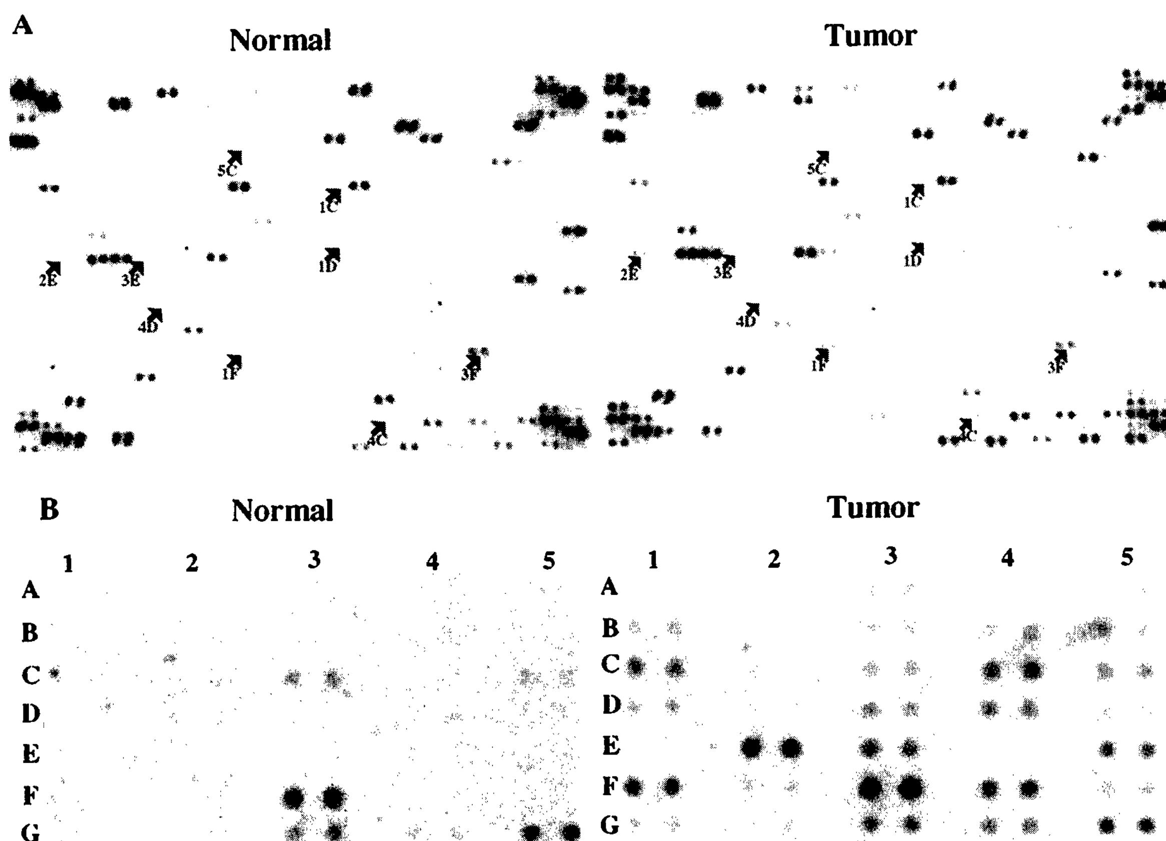


Fig. 1 Representative results of DMH from one patient. The initial screening (*A*) and the corresponding subarray (*B*) were shown with some of the hypermethylated clones later dotted on the subarray identified with their X and Y coordinates. PCR products of CpG island tags were dotted onto membranes hybridized first with radiolabeled normal amplicons as described in the text. The same membranes, or duplicate membranes, were later hybridized with tumor amplicons. Each CpG island tag is represented by two parallel dots to differentiate specific hybridization signals from nonspecific background signals, which generally appear as scattered single dots. Five to six sets of positive controls were dotted on the four corners of the arrays to serve as orientation markers and for comparison of hybridization signal intensities.

This factor was applied to normalize tumor tag volumes. For tags with preexisting methylation in normal tissue, the normal tag volume was subtracted from the normalized tumor volume. For tags without preexisting methylation in the normal tags, the normalized tumor volume was used directly. Statistical analyses were performed using the SigmaStat software (version 2.0). The hypermethylation differences among different groups of tumor grades were determined by the unpaired t test and by the Mann-Whitney rank sum test when the data failed the normality test. The difference was considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Primary Screening of DMH in Breast Tumors. DMH was initially applied to 28 paired breast tumor and normal samples using an array panel containing 1104 CpG island tags. Fig. 1*A* shows representative results of DMH screening in paired normal and tumor samples of patient 103. On the basis of the visual inspection, hypermethylated sequences were identified in breast tumors, showing detectable hybridization signals in CpG island tags probed with tumor amplicons but not in the same tags probed with normal amplicons (see examples indicated by *arrows*). This is because methylated *Bst*UI sites in tumor DNA were protected from restriction within CpG island sequences, which were then amplified by linker-PCR and hybridized to the corresponding tags. The same sites, however, were unmethylated or partially methylated in normal DNA and were restricted by *Bst*UI; therefore, no hybridization signals were detected in the arrays. Some of these hypermethylated CGI island tags were confirmed in the subsequent secondary screening (Fig. 1*B*).

In addition, we observed a few CpG island tags that were detected by normal amplicons (*i.e.,* preexisting methylation) but showed greater signal intensities when probed with tumor amplicons (*e.g.,* CpG island tags in the *lower right-hand corner* in Fig. 1*A*). These sequences usually exhibited more prominent hybridization signals among all of the tags, likely representing abundant copies of GC-rich ribosomal DNA, as described previously in the cell line study (10). Methylation of ribosomal DNA has been observed previously in normal cells (14) but shown to increase to a greater extent in breast tumors (15). Another possibility is that the increased copy numbers of normally methylated CpG island loci in tumors are attributable to aneuploidy. Excluding this preexisting condition, the extent of hypermethylation in unmethylated CpG islands was quite variable among patients in this group; close to 9% of the tested

Table 2 A list of positive CGI clones isolated by DMH

CpG island clone[a]	Insert size (kb)	GenBank match	Accession number
HBC-17[b]	0.75		
HBC-19[b]	0.90	*PAX7*	AL021528
HBC-24[b]	1.10	IMAGE:2518953 5′ mRNA sequence	AI928953
HBC-25[b]	0.70		
HBC-27[b]	0.70		
HBC-33	0.85		
HBC-34	0.95	IMAGE:1113203 3′ mRNA sequence	AA604922
HBC-35	1.00		
HBC-36	0.70		
HBC-37	1.00	PAC 163M9	AL021920
HBC-38	0.50	CGI 40c10	Z58446
HBC-39	0.60	EST185442	AA313564
HBC-40	0.95		
HBC-41	0.60	CGI 29h6	Z58110
HBC-42	0.50	CGI 13f7	Z56764
HBC-43	0.80	Genomic clone NH0444B04	AC007392
HBC-44	1.20		
HBC-45	0.50	PAC 29K1	Z98745
HBC-46	0.65	IMAGE:2177671 3′ mRNA sequence	AI500696
HBC-47	0.70		
HBC-48	1.50	BAC clone RG300E22	AC004774
HBC-49	0.50	CGI 40c10	Z58447
HBC-50	0.45		
HBC-51	0.70	*COL9A1 (alt exon1)*	M32133
HBC-52	0.60	IMAGE:2092259 3′ mRNA sequence	AI381934
HBC-53	0.80	Genomic clone NH0444B04	AC007392
HBC-54	0.85		
HBC-55	0.80	*CAVEOLIN-1 (exon2)*	AF095592
HBC-56	0.60		
HBC-57	0.90	*GATA-3 (exon1)*	X55122

[a] HBC clones have been sequenced, and these data and other information are available to the research community on our Web site: www.missouri.edu/~hypermet.

[b] The clone was hypermethylated in a study of breast cancer cell lines (10) and in primary breast tumors in this study.

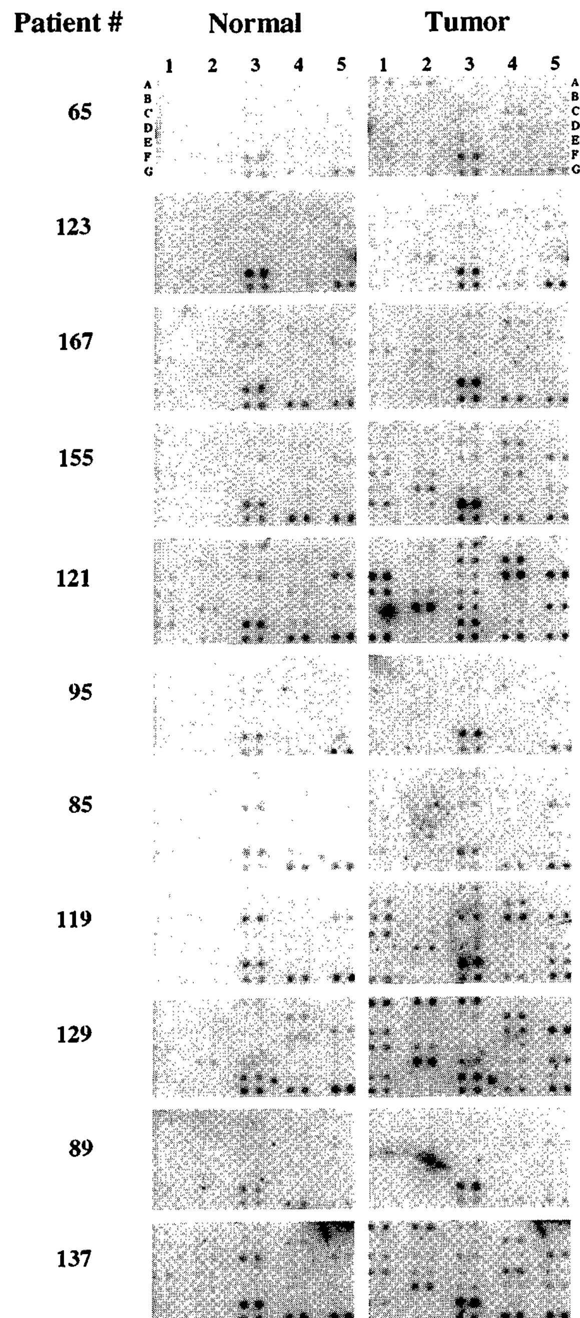
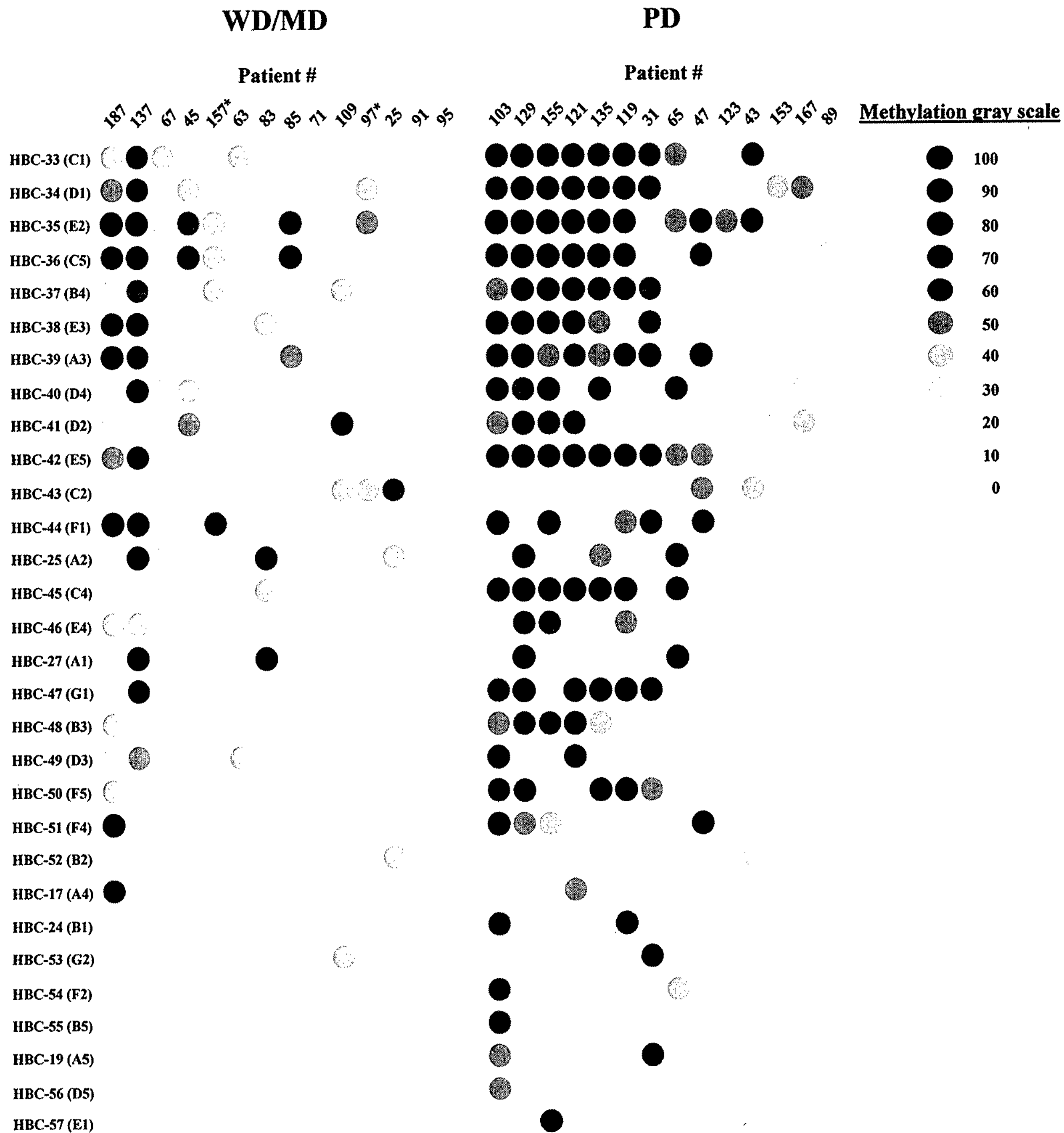


Fig. 2 Identification of hypermethylated CpG island loci by DMH. The 30 CpG island tags shown in this subarray panel were selected from an initial DMH screening of >1000 tags. Five additional tags—coordinates on the X and Y axes are 3C, 3F, 3G, 4G, and 5G—were included as internal controls. CpG island tags were dotted onto membranes in duplicate and probed with radiolabeled amplicons for the normal and breast tumors as indicated. DMH screening from 11 of 28 patients were represented here, and experiments were performed independently twice.

*Bst*UI sites exhibited complete methylation in some breast tumors examined, whereas others had little or no detectable change in the tested sites.

We also noticed that a few tags showed stronger signal intensities when probed with normal amplicons than with tumor amplicons. It is, however, unclear whether these tags represent hypomethylated sequences in the primary tumors analyzed. To our knowledge, specific hypomethylation of normally methylated CpG islands has thus far not been reported in tumors. One possibility is the allelic loss of methylated loci because of chromosomal deletions in tumor cells (9). These tags usually showed stronger hybridization signals and likely contained abundant satellite or repetitive DNA normally methylated but becoming hypomethylated in cancer cells (6, 16). Another possibility is residual normal cells present in tumor specimens, leading to a false-positive identification of hypomethylated sequences. Tissue heterogeneity or contamination in clinical specimens has been a common problem hampering the detection of true genetic or epigenetic alterations in primary tumors. This issue, however, does not apply to the identification of hypermethylated CpG sequences attributable to the gain of additional PCR fragments in tumor amplicons relative to normal amplicons. Because it is less clear whether DMH is suited for the detection of hypomethylated sequences in primary tumors, we



*** WD tumors**

Fig. 3 Hypermethylation pattern analysis of 30 CpG island loci in 28 primary breast tumors. Methylation gray scale shown at *right* represents volume percentile generated by ranking hybridization signal intensities of these tested loci as described in the text. Data from primary tumors were presented according to their tumor grades: WD/MD and PD. Within each group, patients were arranged from *left* to *right* according to their increased methylation propensities. Thirty CpG island loci (on the *left* of the *panel* with their secondary screening coordinates shown in parentheses) were listed from *top* to *bottom* according to their increased methylation scales derived from the primary tumors. Five CpG island loci (HBC-17, HBC-19, HBC-24, HBC-25, and HBC-27) were found to be hypermethylated in breast cancer cell lines, as reported previously by our laboratory.

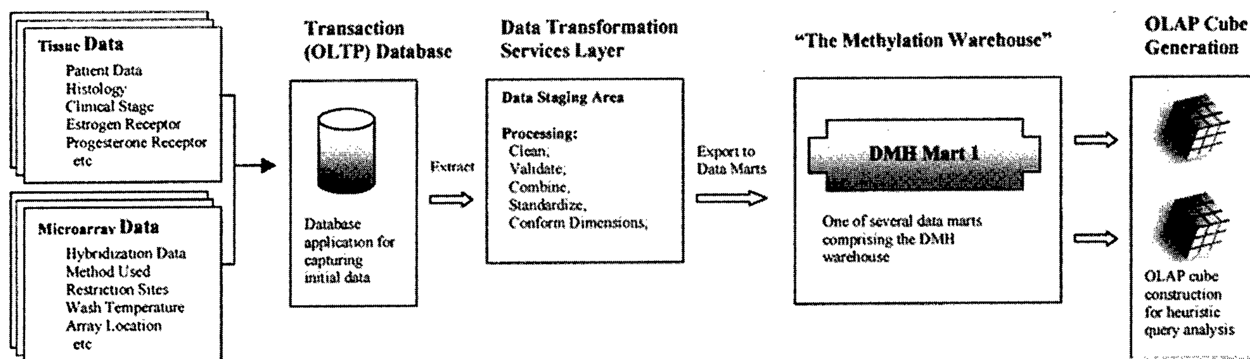


Fig. 4 Diagrammatic representation of the data warehouse used to store and query methylation data. Compared with traditional On-line Transactional Processing (*OLTP*) relational databases, one can visualize patterns across libraries of experiments more effectively using a data warehouse architecture and an On-line Analytical Processing (*OLAP*) browser.

focused on the hypermethylation findings in our subsequent analyses.

Sequence Characterization of CpG Island Tags. Thirty CpG island tags, positive for hypermethylation in the primary screening, were selected for further characterization. DNA sequencing results showed that 9 of these tags contained sequences identical to known cDNAs, *PAX7* (5′ end), *Caveolin-1* (exon 2), *GATA-3* (exon 1), and *COL9A1* (exon 1), and 5 expressed sequence tags (AI928953, AA604922, AA313564, AI500696, and AI381934), as shown in Table 2. This finding is consistent with that of Engelman *et al.* (17), where they also observed CpG island methylation in the *Caveolin-1* gene in breast cancer cell lines. Five CpG island tags, HBC-17, HBC-19, HBC-24, HBC-25, and HBC-27, found to be hypermethylated in breast cancer cell lines as reported previously (10, 18), were also identified in this study. The remainder 25 tags were numerically assigned as HBC-33 through HBC-57, following the previous series of studies mentioned above.

Secondary Screening of DMH in Breast Tumors. As shown earlier in Fig. 1*B*, the 30 CpG island tags were rearrayed for secondary DMH screening in the patient group to confirm their hypermethylation status (see representative results in Fig. 2). Five additional tags–coordinates on the X and Y axes are 3C, 3F, 3G, 4G, and 5G–exhibit no hybridization intensity differences among a few of the breast tumors tested in the primary screening were chosen as internal controls. Again, most normal controls showed few or no detectable hybridization signals at the tested loci, whereas the corresponding breast tumors exhibited various degrees of hybridization intensities, reflecting the differences in CpG island hypermethylation.

To semiquantify the methylation differences, hybridization signal intensity for each CpG island tag was measured using the volume review protocol of ImageQuant software as described in "Materials and Methods." From Fig. 2, it is clear that dot intensities of the internal controls sometimes varied among patients or between a patient's paired tumor and normal samples, likely because of tissue heterogeneity or tumor aneuploidy. Therefore, internal control volume ratios were tested, and two with close volume ratios were selected for normalization. The adjusted tumor volumes were used for clinical correlation in this patient group.

CpG Island Hypermethylation and Tumor Grades. Statistical analysis revealed that CpG island hypermethylation was associated with histological grades of breast tumors (P = 0.041). To aid us in visualizing differences in CpG island hypermethylation among different tumor grades, we devised a gray scale by categorizing tumor methylation volumes into percentiles as depicted in Fig. 3. The PD (3) group exhibited more frequent and extensive hypermethylation at the loci tested than their MD/WD (3) counterparts did; half of the 14 PD tumors showed extensive hypermethylation at multiple loci (>10), whereas only 2 of the 14 MD/WD tumors showed hypermethylation at these loci. Moreover, the greatest degrees of differences were seen at loci HBC-42, HBC-45, and HBC-47 that were frequently hypermethylated in PD tumors but not in MD/WD. This result suggests that patients with more advanced disease status are prone to methylation alterations. It should be noted that some of the patients showed little or no changes of methylation at the loci tested. This indicates that progression of some tumors may be independent of this epigenetic event, or the alteration could occur in later stages of tumor development in such patients. No association of hypermethylation with other clinical parameters was found in this study.

The finding of epigenetic differences in breast tumor grades may have an important implication. We can envisage a mechanism whereby, in patients with methylator phenotype, methylated CpG islands could progressively accumulate during tumor development. As a result of CpG island hypermethylation, critical tumor suppressor genes may become silenced, leading to some cells with growth advantage. Clonal expansion as well as an accumulation of more CpG island hypermethylation would further promote the malignant potential of these cells. Therefore, differential methylation patterns observed in various clinical specimens may reflect different stages or types of cancer. In our case, the most common methylation of CpG island loci (*e.g.*, HBC-33, HBC-34, HBC-35, and HBC-36) observed among different tumor grades likely occurs early during tumor development, whereas methylation groups (*e.g.*, HBC-42, HBC-45, and HBC-47) observed preferentially in the PD group, but not in the WD/MD groups, occur in later stages.

This early study in a small panel of breast cancer patients has proven that DMH is useful for surveying changes of meth-

ylation patterns in cancer. This achievement opens up an unprecedented opportunity for full-scale development of DMH for population-based analysis. Because DMH can be readily reconfigured into a high-throughput, microarray-based assay, this makes possible the detection of CpG island hypermethylation at the whole genome level.

Methylation Data Warehouse. With ~45,000 CpG islands in the human genome, deciphering specific epigenetic signatures in primary tumors can be a daunting task. We have developed a data warehouse, an advanced information system that verifies, cleans, and stores large quantities of methylation data generated from high-throughput DMH experiments. We have also developed a model of data visualization and schema to support this large-scale study. Sophisticated visualization tools are needed to improve knowledge transfer to researchers. We have implemented an On-Line Transactional Processing system that captures DMH raw data, which are then processed by a data transformation services layer for population of the data warehouse (Fig. 4). An On-Line Analytical Processing browser has also been implemented to help researchers perform heuristic queries and view patterns among microarray experiments. The data collected can be readily retrieved to analyze patterns of methylation in tumor samples and to correlate those changes with clinicopathological parameters of patients.

In conclusion, our results demonstrate the power of DMH with hybridization arrays to rapidly generate information on patterns of differentially methylated CpG islands that may then be moved on to population-based studies of the epigenotype-phenotype relationship.

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17β-Hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters

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Summary The expression of 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 and type 2 was examined immunohistochemically in 111 invasive ductal carcinomas, and correlated with various clinicopathological parameters. This study investigates local regulatory mechanisms of oestrogens in human breast carcinoma. 17β-HSD type 1 was immunolocalized in carcinoma cells of 68 out of 111 invasive ductal carcinoma cases (61.3%). 17β-HSD type 2 immunoreactivity was not detected in all cases examined. A significant inverse correlation was observed between the immunohistochemical expression of 17β-HSD type 1 and histological grade of the carcinoma ($P < 0.02$). There was a significant correlation between 17β-HSD type 1 and oestrogen receptor (ER) labelling index (LI) ($P < 0.05$). In addition, carcinoma cells expressing immunoreactive 17β-HSD type 1 were frequently positive for ER. 17β-HSD type 1 was also correlated with progesterone receptor (PR) LI ($P < 0.05$). There was a significant inverse correlation between 17β-HSD type 1 and Ki-67 LI ($P < 0.0001$). No significant correlations were detected between 17β-HSD type 1 and other clinicopathological parameters, including patient age, menopausal status, stage, tumour size, lymph node status and prognosis. This study suggests that 17β-HSD type 1 plays an important role in the regulation of in situ oestradiol production in hormone-dependent breast carcinomas. © 2000 Cancer Research Campaign

Keywords: 17β-HSD; breast; carcinoma; human; immunohistochemistry; oestrogen

Breast carcinoma is one of the most common malignancies in women worldwide. Human breast tissue is a target for oestrogens. Oestrogens have an important role to play in the development of hormone-dependent breast carcinomas (Thomas, 1984; Vihko and Apter, 1989), and oestrogen receptor (ER) status is well-known to affect the prognosis of patients with breast neoplasms (Rose et al, 1983). 17β-hydroxysteroid dehydrogenase (17β-HSD) catalyses the reversible interconversion of oestrogens. Recently, isozymes of 17β-HSD have been cloned, and it has been demonstrated that 17β reduction and oxidation of oestrogens are catalysed by different 17β-HSD isozymes. 17β-HSD type 1 catalyses the conversion of inactive oestrogen, oestrone (E1), to biologically active oestrogen, oestradiol (E2) (Peltoketo et al, 1988; Luu-The et al, 1989; Gast et al, 1989), while 17β-HSD type 2 catalyses the conversion of E2 to E1 (Wu et al, 1993). 17β-HSD type 1 and type 2 regulate the tissue level of E2 and modulate oestrogenic actions in oestrogen target tissues. Therefore, examining the expression of 17β-HSD type 1 and type 2 in human breast carcinoma is critical in obtaining a better understanding of the local regulation of oestrogenic actions in human breast carcinoma.

Recently, immunohistochemical studies of 17β-HSD type 1 have been reported in human breast carcinoma by Poutanen et al (1992*a*) and Sasano et al (1996). However, the number of patients examined was relatively limited in these reports, and the relationship to clinicopathological parameters and the biological significance of 17β-HSD type 1 are still unclear. In addition, little is known about 17β-HSD type 2 in human breast carcinoma. Therefore, in this study, we immunohistochemically examined the expression of 17β-HSD type 1 and type 2 in 111 human breast carcinomas, and correlated these findings with various clinicopathological parameters, in order to study the local regulatory mechanisms of oestrogens in human breast carcinoma.

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MATERIALS AND METHODS

Patients and tissues

A total of 111 specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1984 to 1989 at the Department of Surgery, Tohoku University Hospital, Sendai, Japan. The mean age was 52 years (range 27–82). All the patients examined had not received irradiation or chemotherapy prior to surgery. The clinical data, including patient age, menopausal status, stage according to UICC TNM classification (1987), tumour size and lymph node status, were retrieved from patient charts. The histological grade in each specimen was evaluated by three of the authors (TS, TM and NA), based on the modified method of Bloom and Richardson (1957) according to Elston and Ellis (1991). The mean follow-up time was 110 months (range 13–157). Disease-free survival data were available for all patients.

All specimens were routinely processed (10% formalin-fixed and paraffin-embedded) at the Department of Pathology, Tohoku University Hospital, Sendai, Japan.

The resulting methylation data and patients' clinicopathological features are summarized in Table 1. An overall increase of rDNA methylation was seen in ~80% of the breast tumours examined. The mean percent methylation of breast tumours was significantly higher than that of normal breast tissue samples ($P < 0.0001$). The percent rDNA methylation was also significantly higher in ER-negative tumours than in ER-positive tumours ($P < 0.0273$), and in moderately or poorly differentiated tumours in comparison to well-differentiated tumours ($P < 0.0475$). As we further subdivided groups by ER and PR combined expression, the poorly differentiated tumours had significantly higher rDNA methylation than the moderately or well differentiated tumours within the ER-positive and PR-positive subgroup ($P < 0.0376$). rDNA methylation was essentially the same between moderately and poorly differentiated tumours in the ER-negative and PR-negative subgroup ($P < 0.5828$; none of the well differentiated tumours belonged to this subgroup). When the diagnosed age of 50 years old was used as the cutoff, tumours from younger patients (≤ 50 years old) had higher rDNA methylation though the observed differences were of borderline statistical significance (*P*-value of 0.0743). Interestingly, this inverse association between age at diagnosis and rDNA methylation was observed in most of the tumour subgroups as shown in the 'Age' and '% rDNA methylation' columns in Table 1. The percent rDNA methylation was not associated with tumour TNM classifications, PR negativity, or tumour mitotic frequency.

DISCUSSION

This study presents evidence that, in addition to single-copy CpG islands, abundant rDNA sequences are another type of methylation substrate in breast cancer. Our data indicated that increased rDNA methylation was often found in subgroups of patients with tumour undifferentiation and with younger diagnosed age (≤ 50 years old). Interestingly, rDNA hypermethylation was also correlated with ER negativity ($P < 0.0273$). Previous studies have shown that hypermethylation of the *ER* CpG island was associated with the lack of ER expression in ER-negative breast cancer cells (Ottaviano et al, 1994) and in 25% of ER-negative tumours (Lapidus et al, 1996). These studies also indicated that methylation silencing of the *ER* gene might be a primary cause responsible for some breast cancer patients subsequently becoming insensitive to anti-oestrogen therapies. Together with these previous results, we hypothesize that hypermethylation of rDNA and the *ER* CpG island can be a related event in breast cancer. If this assumption were further proven, then the finding could support a generalized mechanism governing the epigenetic event. Since DNA hypermethylation may arise as a stochastic process in tumour cells as indicated earlier, abundant copies of rDNA can be more available than single-copy CpG islands in this chance event.

We, therefore, reason that rDNA is a potential marker for tumours having a greater propensity to methylate their genome (i.e. hypermethylator phenotype). In the aforementioned clinical correlation, the random methylation process can simultaneously occur in both rDNA and the *ER* CpG island in this subgroup of breast tumours. In other breast tumours with a hypermethylator phenotype, aberrant methylation may have already occurred in abundant rDNA, but not yet in the *ER* CpG island. Thus, can the status of hypermethylated rDNA predict this type of tumour having a high likelihood of developing an ER-negative phenotype due to subsequent hypermethylation of the *ER* CpG island? Future methylation studies of rDNA together with *ER* and other gene CpG islands in breast cancer are needed to address this question.

In conclusion, we have shown for the first time that rDNA hypermethylation occurs in breast tumours, and may be an important marker for this epigenetic event in neoplasia. Our finding highlights the need for further investigations of rDNA hypermethylation and its relationship to the development of breast carcinoma.

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Table 1 Clinicopathological features of patients with infiltrating ductal carcinoma of the breast.

Patient characteristics	*n*	Age (mean ± SD)	%rDNA methylation (mean ± SD)	*P*-value[a]
Normal vs. tumour				
Normal breast tissue	10	57.9 ± 15.2	41.0 ± 10.2	**0.0001**
Tumour breast tissue	58	58.4 ± 15.7	62.4 ± 14.4	
Age at diagnosis (years)				
<50	20	41.8 ± 4.5	67.1 ± 13.5	NS (0.0743)
≥50	38	67.2 ± 11.8	60.0 ± 14.4	
Oestrogen receptor (ER)				
Positive	31	64.1 ± 15.7	58.5 ± 16.0	**0.0273**
Negative	27	51.8 ± 13.0	67.0 ± 10.8	
Progesterone receptor (PR)				
Positive	20	63.8 ± 16.4	59.5 ± 14.9	NS (0.1430)
Negative	34	54.9 ± 14.3	65.5 ± 13.8	
Combined ER/PR status				
ER+/PR+	22	64.4 ± 16.5	59.0 ± 15.1	NS (0.0848)
ER+/PR–	9	63.4 ± 14.4	57.2 ± 19.0	
ER–/PR–	26	51.9 ± 13.3	66.9 ± 10.0	
Mitotic frequency				
<20 HPF[b]	37	60.7 ± 16.6	61.4 ± 13.8	NS (0.1104)
≥20 HPF	15	52.0 ± 13.8	68.2 ± 13.6	
Tumour differentiation				
In all the tumor tissues				
WD[c]	3	76.0 ± 17.3	49.2 ± 18.3	**0.0475**
MD/PD	50	56.8 ± 15.0	64.8 ± 12.7	
In ER+/PR+ subgroup				
WD/MD	16	65.8 ± 16.1	57.4 ± 13.8	**0.0376**
PD	4	59.5 ± 17.9	73.8 ± 8.70	
In ER–/PR– subgroup				
MD	12	54.0 ± 14.7	68.2 ± 7.1	NS (0.5828)
PD	14	50.1 ± 12.2	65.7 ± 13.6	
TNM classification[d]				
I	9	63.4 ± 14.6	62.6 ± 14.7	NS (0.4966)
II	28	58.6 ± 15.3	63.7 ± 13.5	
III	7	52.0 ± 10.9	61.5 ± 16.5	
IV	5	51.6 ± 18.7	73.2 ± 9.30	

[a]Statistical analyses performed on the rDNA methylation values vs. patient characteristics using PROC TTEST or PROC GLM (SAS System, Cary, NC). Statistically significant ($P < 0.05$). NS, not significant. [b]HPF, high power field, 40×. [c]WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated. All the WD were in the ER+/PR+ subgroup. [d]Classification according to the TNM system (Beahrs, 1989).

(Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Washing was performed once for 20 min in 0.1% sodium dodecyl sulphate (SDS)–0.5× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and thrice for 20 min each in 0.1% SDS–0.2× SSC at 70°C. The hybridized membranes were subjected to image analysis with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Band intensities were quantified using the densitometric function of the ImageQuant software (Molecular Dynamics). Methylation was expressed as the percentage of the intensity of the methylated fragments to the combined intensities of all the fragments in *Hpa*II sample lanes as depicted in Figure 1B.

Statistical analyses

The percent rDNA methylation values and the patient ages were reported as mean ± standard deviation (s.d.). The association between percent rDNA hypermethylation data and patients' clinicopathological data were analysed using the SAS procedure PROC TTEST for comparisons having two variables and PROC GLM for comparisons having three or more variables (Release 6.12, SAS Institute, Cary, NC, USA). Statistical significance was established as $P < 0.05$.

RESULTS

Methylation analysis was conducted by Southern hybridization in 58 primary breast tumours and ten normal controls using probes spanning the transcribed domain of rDNA (Figure 1A). A total of 280 *Hpa*II/*Msp*I sites located within this region were examined. Representative results are shown in Figure 1B. In control samples, the pattern (fragments with size < 1 kb) of methylation-sensitive *Hpa*II restriction was largely the same as that of methylation-insensitive *Msp*I restriction, indicating that the majority of these sites within the transcribed domain were unmethylated. Some fainter fragments appeared as smears (> 1 kb) in the *Hpa*II-restricted lanes, suggesting a minor proportion of these sites were methylated and protected from restriction, consistent with a previous observation in normal cells (Brock and Bird, 1997). In tumour samples, the patterns between methylation-sensitive and -insensitive restrictions were often different. The predominant fragments of the *Hpa*II-restricted fragments shifted into regions of higher molecular weights (> 1 kb); a smear of varying length and band intensity was seen in these regions due to the differing degrees of methylation in tumour samples. The *Msp*I-digested fragments in tumours remained essentially similar to those of the normal control samples.

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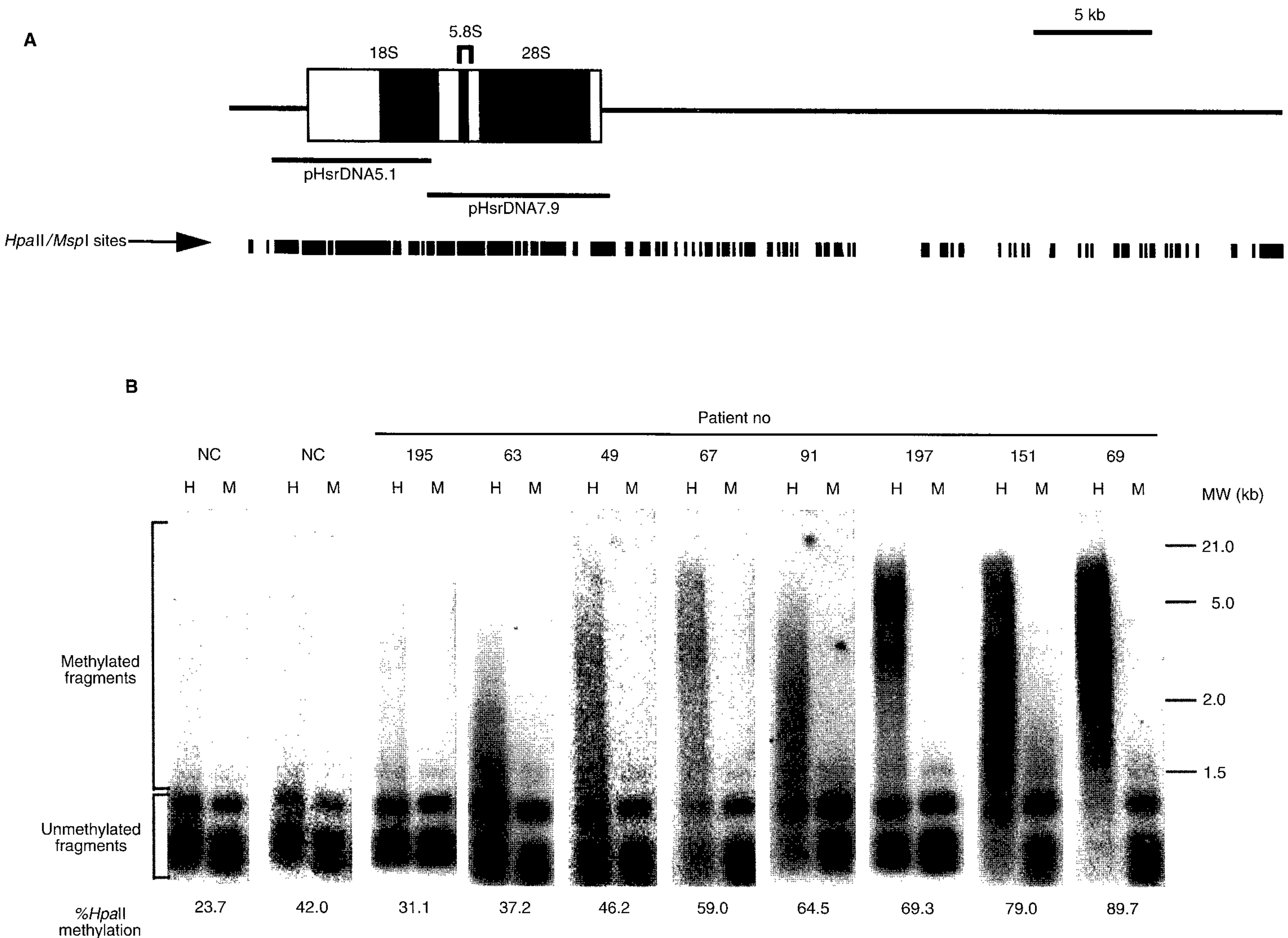

Figure 1 (**A**) Map of a single ribosomal DNA repeat unit showing the positions of probes (pHsrDNA5.1 and pHsrDNA7.9) used in Southern analysis. The transcriptional units 18S, 5.8S and 28S RNA are shown in filled boxes and the internal and external transcribed spacers are represented in unshaded boxes. Vertical bars mark the relative positions of *Hpa*II/*Msp*I (CCGG) sites in the repeat unit. (**B**) Methylation analysis of ribosomal DNA in primary breast tumours and normal breast tissues (NC). Genomic DNA was digested with methylation-sensitive *Hpa*II (H) or its methylation-insensitive isoschizomer *Msp*I (M), and subjected to Southern hybridization using the combined pHsrDNA5.1 and pHsrDNA7.9 as probes. The methylated and unmethylated fragments are indicated at left and molecular weight markers are shown at right. Eight patient tumour samples with increasing % methylation were selected and shown with their respective densitometric data

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Hypermethylation of ribosomal DNA in human breast carcinoma

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Summary We examined the methylation status of the transcribed domain of ribosomal DNA (rDNA) in 58 patients with breast cancer. The mean percent of methylation was significantly higher in breast tumours than that of normal control samples ($P < 0.0001$). This increased rDNA methylation was associated with oestrogen receptor non-expression ($P < 0.0273$) and with moderately or poorly differentiated tumours as compared to well differentiated tumours ($P < 0.0475$). Our results suggest that rDNA can be a useful marker for monitoring aberrant methylation during breast tumour progression. © 2000 Cancer Research Campaign

Keywords: DNA hypermethylation; ribosomal DNA; breast cancer

In human cancer, DNA hypermethylation is known to occur in CpG islands, which are 1- to 2-kb GC-rich regions frequently located within the 5′ ends of about 60% of all genes (Laird and Jaenisch, 1994). This type of epigenetic mutation has been shown to be associated with transcriptional silencing of tumour suppressor genes in neoplasia (Baylin et al, 1997). The abnormal event is generally accepted as a stochastic process in tumour cells with a hypermethylator phenotype (Pfeifer et al, 1990; Jones, 1996; Huang et al, 1999). The random process may occur at CpG sites within the 5′ regulatory regions of critical tumour suppressor genes. The resulting progressive silencing of transcription can provide these cells with a greater proliferative advantage (Jones, 1996). In addition to classical genetic mutations, DNA hypermethylation plays a significant role in promoting tumorigenesis.

Abundant ribosomal DNA shares some characteristics with single-copy CpG islands. The entire 13.3-kb transcriptional domain of ribosomal DNA (rDNA) is GC-rich, but is much longer than a typical 1- to 2-kb CpG island (Worton et al, 1988). In normal human cells, the rDNA transcribed domain is predominately unmethylated, and has been associated with active transcription of 18S, 5.8S and 28S RNA subunits (Dante et al, 1992; Gonzalez et al, 1992). Juxtaposed to the 3′ end of the transcribed domain is a low GC-containing non-transcribed spacer (30-kb) known to contain methylated CpG sites (Brock and Bird, 1997). Approximately 400 copies of rDNA per haploid genome are located on the short arms of human acrocentric chromosomes (Worton et al, 1988). These repeat units are arranged in head-to-tail arrays with each chromosome cluster containing approximately 80 copies (Sakai et al, 1995).

Since both rDNA and CpG islands share similar properties, we sought to determine whether rDNA is subject to aberrant methylation in breast cancer. Methylation analysis was performed by Southern hybridization using the entire transcribed region as a probe in a group of patients with infiltrating ductal carcinomas. The resulting rDNA methylation data were used to examine their association with patients' clinicopathological parameters.

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PATIENTS AND METHODS

Patients and samples

Breast tumour specimens were obtained from 58 patients undergoing partial or complete mastectomies at the Ellis Fischel Cancer Center (Columbia, MO, USA). Specimen collection and tissue analyses were approved by the Institutional Review Board of the University of Missouri Health Science Center. Clinicopathological parameters and TNM (Tumour-Nodal-Metastasis) classification were performed using standard criteria (Beahrs, 1989). All tumours were classified as infiltrating ductal carcinomas. The oestrogen receptor (ER) and progesterone receptor (PR) status of tumour tissues was determined by either the dextran-coated charcoal assay (negativity defined as ≤ 3 fmol mg^{-1} ligand bound protein) or the immunoperoxidase technique (negativity defined as $\leq 20\%$ of tumour nuclei stained positive). Non-neoplastic breast tissue was also obtained from ten study subjects and used as 'normal controls'. High-molecular-weight DNA was isolated using the QIAamp Tissue Kit™ (Qiagen Inc, Chatsworth, CA, USA).

Methylation analysis by Southern hybridization

Genomic DNA (approximately 2.5 μg) from breast tissues was digested to completion with methylation-sensitive *Hpa*II (cuts C↓CGG, but not C^{m}CGG; m: methylated) or its methylation-insensitive isoschizomer *Msp*I (cuts both C↓CGG and C↓^{m}CGG). The restriction products were separated on 1.0% agarose gels and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labelled pHsrDNA5.1 and pHsrDNA7.9 probes (Figure 1A) at 70°C in 10 ml of High Efficiency Hybridization solution (Molecular Research, Inc., Cincinnati, OH, USA). Probes were radiolabelled using the Multiprime DNA Labelling System

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Report

Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas

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Key words: breast cancer, CpG island, DNA hypermethylation, Wilms' tumor suppressor gene

Summary

CpG island hypermethylation is known to be associated with transcriptional silencing of tumor suppressor genes in neoplasia. We have previously detected aberrantly methylated sites in the first intron of the Wilms' tumor suppressor (*WT1*) gene in breast cancer. In the present study, we extended the investigation to a CpG island located in the promoter and first exon regions of *WT1*. Methylation of this CpG island was found to be extensive in MCF-7 and MDA-MB-231 breast cancer cells, as well as in 25% (five of 20 patients) of primary breast tumors. While levels of the known 3.0-kb *WT1* mRNAs were decreased or not detected in these cell lines, the expression could be partially restored following treatment with a demethylation agent, 5-aza-2′-deoxycytidine. Surprisingly, a novel 2.5-kb *WT1* transcript was expressed at high levels in both untreated and treated MDA-MB-231 cells. This novel transcript was likely a *WT1* variant missing the first exon, and therefore escaped the methylation control present in the normal transcript. Our study implicates the future need to investigate the significance of this aberrant transcript as well as the role of *WT1* CpG island hypermethylation in breast neoplasia.

Introduction

An increase in the cellular capacity to selectively methylate genomic DNA has been observed in breast cancer cells [1]. The event usually occurs at the fifth carbon position of a cytosine located 5′ to a guanine in what is known as a CpG dinucleotide [2, 3]. The dinucleotides are clustered frequently in 1–2-kb regions, known as CpG islands, around the promoters and the first exons of genes [4]. Aberrant DNA methylation in these regions has been shown to provide an alternative pathway, in addition to deletions or mutations, for the silencing of tumor suppressor genes in a variety of cancer types [5–9]. In breast cancer, this epigenetic phenomenon is associated with the inactivation of the estrogen receptor (ER) gene expression in ER-negative cancer cells [1, 10] and with resistance to hormonal therapies in some patients [11].

Our laboratory has been developing strategies to identify novel CpG islands associated with aberrant methylation during the progression of breast neoplasia. Using the methylation-sensitive restriction fingerprinting technique [12], we previously identified a DNA fragment containing the first intron of the Wilms' tumor suppressor (*WT1*) gene, which was frequently hypermethylated in primary breast tumors but unmethylated in the normal breast tissue of the same patients. Since the 5′ end of this gene met the criteria of a CpG island [13], we hypothesized that the *WT1* gene is a candidate for inactivation via methylation silencing in breast cancer.

WT1 was first identified as a tumor suppressor gene by positional cloning on chromosome 11p13 in association with Wilms' tumor, a nephroblastoma common to children [14, 15]. The *WT1* gene is known to express four splice variants, each approximately 3 kb in length [14, 15]. As nuclear transcription factors, the *WT1* proteins modulate expression of a variety of growth factors and their receptors [16]. Functional analysis has demonstrated that the *WT1* gene plays

a crucial role in the mesenchymal-to-epithelial cell transition in the embryonic kidney as well as in other developing tissues of mesothelial origin [17]. Although loss of *WT1* expression was initially associated with the development of Wilms' tumor, additional reports have correlated abnormal *WT1* expression with leukemias, melanomas, mesotheliomas, and ovarian cancers [18–21].

Recently, *WT1* protein was found to be present in normal breast tissues, but was absent or greatly reduced in a subset of primary breast tumors [22]. These findings, coupled with our previous methylation analysis near the 5′ end of the *WT1* gene [12], suggested that methylation silencing is a potential mechanism for *WT1* gene inactivation in breast cancer. In this study, we show that hypermethylation of the *WT1* CpG island was extensive in MCF-7 and MDA-MB-231 breast cancer cells, and in a subset of primary breast tumors. This aberrant event was associated with transcriptional suppression of normal *WT1* transcripts in the cell lines. In addition, we detected a novel transcript that was not subject to methylation silencing and was expressed at a high level in MDA-MB-231 cells.

Materials and methods

Cell culture and tissue samples

The MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from Dr V.C. Jordan (Northwestern University); ZR-75-1 and Hs578t breast cancer cells were obtained from the American Type Culture Collection. MCF-7, MDA-MB-231 and Hs578t cells were routinely maintained in Eagle's minimum essential medium containing 10% fetal bovine serum. ZR-75-1 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. 5-aza-2′-deoxycytidine (deoxyC) was obtained from Sigma, and freshly prepared in distilled water. Cells were plated at a density of 2×10^4 cells/cm^2 and treated continuously with 0.75 µM deoxyC for two and six days. Control cultures were maintained in the absence of deoxyC. Breast tumor specimens were obtained from patients undergoing mastectomies or biopsies at the Ellis Fischel Cancer Center (Columbia, MO). Adjacent, non-neoplastic breast tissue was obtained from the same patient to serve as a normal control. The patient study was approved by our institutional review board (IRB# 5326).

WT1 probe preparation

A probe, WT1–6 (425 bp), was obtained from *WT1*'s first exon (positions 361–785; GenBank accession no. ×51630) using PCR with the following primers: 5′-TCC GGG TCT GAG CCT CAG CAA A (sense strand); 5′-CCC GTC CAT CCC GCG CAA TCC (antisense strand). The second probe, WT1-7, was an amplified fragment (481 bp) between exons 7 and 10 of the gene using primer sequences previously described [23]: 5′-GGC ATC TGA GAC CAG TGA GAA (sense strand) and 5′-GAG AGT CAG ACT TGA AAG CAG T (antisense strand). PCR products were subcloned into the pCR2.1 cloning vector (Invitrogen) and the inserts were confirmed by DNA sequencing. WT1–6 and WT1–7 were ^{32}P-labeled by the random primer method (Amersham) and used as probes for southern and northern hybridization, respectively.

Southern hybridization

For the analysis of *WT1* CpG island methylation status, 5 µg of genomic DNA from cell lines or patients' specimens were digested to completion with *Mse* I alone or subsequently with methylation-sensitive *Bss*H II, *Sac* II, or *Bst*U I (New England Biolabs). For the structural analysis of the *WT1* CpG island in MDA-MB-231 cells, 5 µg of genomic DNA from this cell line was independently digested with *Pvu* II, *Taq* I, and *Xho* I (New England Biolabs). The digestions were performed as per the supplier's protocols. The restriction products were electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane (Schleicher and Schuell). Membranes were hybridized with the WT1-6 probe. Prehybridization, hybridization, and washing were performed essentially as previously described [24]. Membranes were exposed to Kodak BioMax film in the presence of an intensifying screen for seven days at −70°C. Alternatively, membranes were exposed in a PhosphorImager (Molecular Dynamics) and the results were analyzed using the Image Quant software (Molecular Dynamics).

Northern hybridization

Total RNA was isolated from the breast cancer cell lines using RNeasy total RNA kit™ (Qiagen). Twenty micrograms of RNA were electrophoresed on a 1.4% agarose gel in the presence of 2.2 mM formaldehyde and transferred to a nylon membrane. The membrane

was hybridized with the WT1–7 probe. Hybridization and washing conditions were used as previously described [24]. The membrane was exposed to Kodak BioMax film for eight days at −70°C. Alternatively, the membrane was exposed in a PhosphorImager and the results were analyzed using the Image Quant software. The same membrane was reprobed with a ^{32}P-labeled *β-actin* cDNA probe (1.1 kb) to compare loaded RNA levels. The *β-actin*-probed membrane was similarly examined by PhosphorImager analysis.

Reverse transcription (RT)-PCR

For each sample, 4 μg of total RNA was reverse-transcribed using oligo(dT) primers with the Superscript Preamplification System (Life Technologies). *WT1* primers were designed to amplify an exon 1 fragment (275 bp; sense strand: 5′-TGG GCA GGT AGG GCG CGT TAG GAA; antisense strand: 5′-CGC TCC GGC TTA CGG GTC GTT GG) and a fragment (154 bp) between exons 7 and 8 (sense strand: 5′-AAC GCC CCT TCA TGT GTG C; antisense strand: 5′-GCT GGT CTG AAC GAG AAA ACC TTC). Primer sequences designed for a *β-actin* cDNA fragment (541 bp) were 5′-GTG GGG CGC CCC AGG CAC CA (sense strand) and 5′-GTC CTT AAT GTC ACG CAC GAT TTC (antisense strand). In order to allow for comparison of *WT1* levels between samples, simultaneous PCR amplifications for *WT1* and *β-actin* cDNAs were performed with cDNA (250 ng) in 30 μl volume, containing a pair of *WT1* primers (0.6 μM each) and *β-actin* primers (0.06 μM each), 0.8 units Deep Vent polymerase (New England Biolabs), 200 μM dNTPs, 1 μCi [$\alpha-^{32}$P] dCTP (3000 Ci/mmole; Amersham) in a buffer provided by the supplier. A touchdown PCR program was employed using the cycling parameters: 96°C for 40 s, 64°C −0.5°C per cycle for 30 s, and 50 s at 72°C for 22 cycles. Under this low cycling condition, amplification was expected to be in the linear range of the assay. PCR products were size-fractionated on a 4.5% non-denaturing polyacrylamide gel. After electrophoresis, wet gels were wrapped with plastic film and exposed to Kodak BioMax film at −70°C. Autoradiography for *WT1* and *β-actin* cDNAs was carried out for 15 h. The levels of *WT1* cDNAs were normalized with the level of *β-actin* product band in the respective samples. RT-PCR was independently performed at least two times with different RNA sources.

Results

Methylation analysis of the WT1 CpG island in breast cancer cell lines

We examined the methylation status of the *WT1* promoter and first exon regions in MCF-7 and MDA-MB-231 cells by southern hybridization. As shown in Figure 1a, this region displays characteristics of a CpG island, being highly GC-rich with a G+C content of 70% and having a CpG/GpC ratio of 74%. The region includes many recognition sites for methylation-sensitive endonucleases, including two *Bss*H II, one *Sac* II, and 12 *Bst*U I sites. These sites are located within a 1.6-kb fragment flanked by two recognition sequences for *Mse* I. *Mse* I cuts genomic DNA into small fragments (once per ~140 bp), leaving CpG islands generally intact [25]. As expected, digestion of genomic DNA with *Mse* I alone produced a 1.6-kb fragment (Figure 1b, lanes 1, 5, and 9). An additional fragment with slightly less molecular weight than the 1.6-kb fragment was observed in the control sample (lane 1). This may represent a polymorphism, as this smaller band was also detected in breast tissue from patients 67 and 127 (Figure 2). Double digestion of the control DNA with *Mse* I and each of the three methylation-sensitive endonucleases, *Bss*H II (lane 2), *Sac* II (lane 3), and *Bst*U I (lane 4) yielded the expected 1.4−, 0.9−, and <0.2-kb fragments, respectively, demonstrating that these sites were not methylated in the control sample. In contrast, the 1.6-kb *Mse* I-restricted fragment appeared to be protected from the methylation-sensitive restrictions (lanes 6–8) in the MCF-7 samples, suggesting that each of these sites was completely methylated within the *WT1* CpG island of this cell line. A similar southern hybridization result was obtained in the MDA-MB-231 samples (lanes 10–12), except that retention of additional minor fragments with smaller molecular weights in the *Mse* I/*Sac* II and *Mse* I/*Bst*U I digests was observed. This could be attributed to the cellular heterogeneity in the MDA-MIB-231 cell line, showing the presence of a few unmethylated CpG sites in some cells.

Methylation analysis of the WT1 CpG island in primary breast tumors

Methylation analysis by southern hybridization was undertaken on breast tumor samples from 20 patients with infiltrating ductal carcinomas. One methylation-sensitive enzyme, *Bst*U I, was used in the analysis because of the limited amount of available patient

Figure 1a. Schematic representation of the *WT1* CpG island in the promoter and first exon regions. The relative positions of the methylation-sensitive restriction sites for *Bst*U I, *Bss*H II, and *Sac* II as well as other methylation-insensitive restriction sites (*Mse* I, *Pvu* II, *Taq* I, and *Xho* I) are indicated. The relative density of CpG dinucloeotides is also indicated. A fragment spanning the first exon, WT1–6, was used as a probe in southern hybridization. The *Mse* I fragment is approximately 1.6 kb in length. The dotted line indicates DNA sequence not reported in the literature. **Mse* I denotes a polymorphic site observed in some patients.

Figure 1b. Methylation analysis of breast cancer cell lines MCF-7 and MDA-MB-231 as well as normal control tissue by southern hybridization. Genomic DNA (5 μg) was treated (+) or untreated (−) with restriction endonucleases as indicated and subjected to southern hybridization analysis using WT1–6 as the probe. Molecular weight markers (100-bp ladder; Life Technologies) are shown at left.

DNA. Restriction of genomic DNA with this endonuclease also enabled the examination of a greater number of possible methylated cytosines within the *WT1* CpG island (see Figure1a). Genomic DNA samples from breast tumors were digested with both *Mse* I and *Bst*U I, and hybridized with the WT1–6 probe. DNA samples from available matching normal tissues were similarly analyzed. Figure 2 shows representative results for seven of these patients. The double-digested tumor DNA from patients 29, 69, 119, and 127 hybridized to the 1.6-kb fragment, indicating complete methylation of all *Bst*U I sites within the *WT1* CpG island as described earlier. Methylation of the island was not observed in the tumor DNA of patients 133 and 135. Additional smaller-sized fragments were detected in patient 29 due to the presence of unmethylated *Bst*U I sites in some of the patient's tumor cells. The DNA from patient 89 hybridized to a smaller fragment (~1.0 kb), indicating partial methylation of the *WT1* CpG island in this tumor. Methylation of the *WT1* CpG island was not detected in the matching normal samples from these patients, although normal tissue

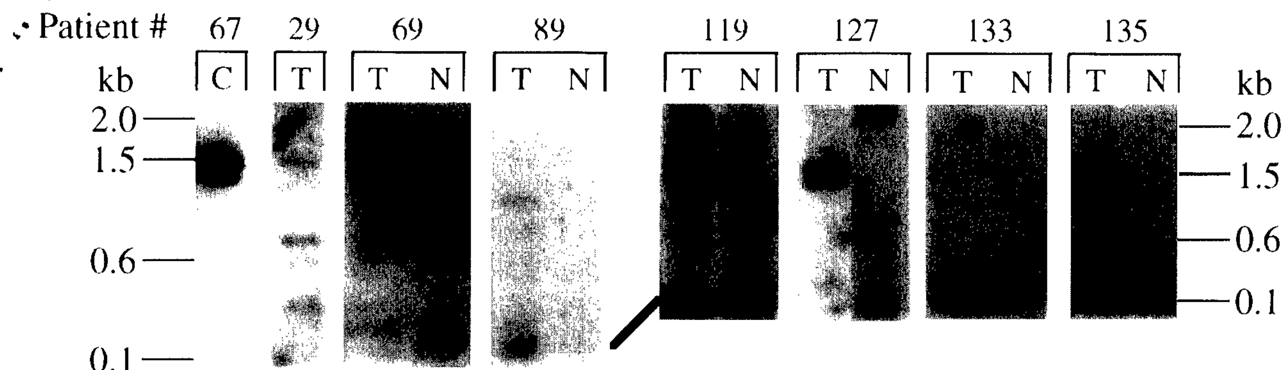

Figure 2. Methylation analysis of the *WT1* gene in primary breast tumors (T) and the matching normal breast tissues (N) from patients by southern hybridization. No normal control was available for patient 29. Genomic DNA (5 μg) was treated consecutively with *Mse* I and *Bst*U I, then subjected to southern hybridization analysis using *WT1*-6 as the probe. C: Control DNA digested with *Mse* I only. Representative molecular weight markers (from the 100-bp ladder; Life Technologies) are shown flanking both sides of the figure.

Table 1. Clinical information and methylation studies of female patients with infiltrating ductal carcinoma of the breast

Patient no.	Age at diagnosis (yr)	Clinical staging[a]	Methylation status of *WT1* in tumor[b]
29	77	I	+
69	37	IIa	+
73	57	IIa	–
81	72	IIb	–
89	83	IV	+
101	91	I	–
103	67	IIa	–
107	47	IIa	–
109	66	IIa	–
111	70	IIIa	–
113	81	IIa	–
117	46	IIIa	–
119	67	IIIa	+
121	52	IIIa	–
123	38	IIb	–
127	35	IIIb	+
129	76	IIIb	–
131	80	ND	–
133	44	IIb	–
135	46	IIa	–

[a]Clinical staging was according to the criteria of the American Joint Committee on Cancer [33].
[b]+, hypermethylation in tumor; –, lack of methylation in tumor (see additional description in the text).

from patient 29 was unavailable for study. The results of this methylation analysis and the patients' clinical information are summarized in Table 1.

Methylation silencing of the WT1 gene in breast cancer cell lines

We next investigated whether the *WT1* CpG hypermethylation correlated with down-regulation of the *WT1* gene in MCF-7 and MDA-MB-231 cells. Cells were treated with a demethylating agent, deoxyC, and patterns of their *WT1* gene expression were determined by northern hybridization. Initial analysis using probe WT1–6 produced non-specific hybridization patterns, and was attributed to this probe's highly GC-rich nature. A second probe, WT1–7, specific to *WT1* exons 7–10 was generated for northern hybridization. WT1–7 detected a band migrating at 3.0-kb corresponding to the size of the previously described *WT1* mRNAs [26] in the control cell lines ZR-75-1 (lane 1) and Hs578t (lane 3). These cell lines exhibited no hypermethylation of the *WT1* CpG island region by prior southern analysis (data not shown), and a 6-day treatment of both cell lines with deoxyC resulted in no change in *WT1* expression. The 3.0-kb transcripts were faintly detectable in the MCF-7 untreated cells (lane 5), and this expression level slightly increased following treatment with deoxyC for six days (lane 7). The partial restoration of *WT1* expression suggests that either the deoxyC dosage was inadequate to effectively demethylate the *WT1* promoter and thereby induce full expression, or that other factors in addition to methylation suppress *WT1* transcription in the MCF-7 cell line. Both untreated and 2-day treated MDA-MB-231 cells expressed no detectable 3.0-kb transcripts (lanes 8 and 9). However, treatment of MDA-MB-231 cells for six days resulted in a greater increase in expression of 3.0-kb transcripts than in MCF-7 cells (lane 10). Surprisingly, a *WT1* mRNA variant with a smal-

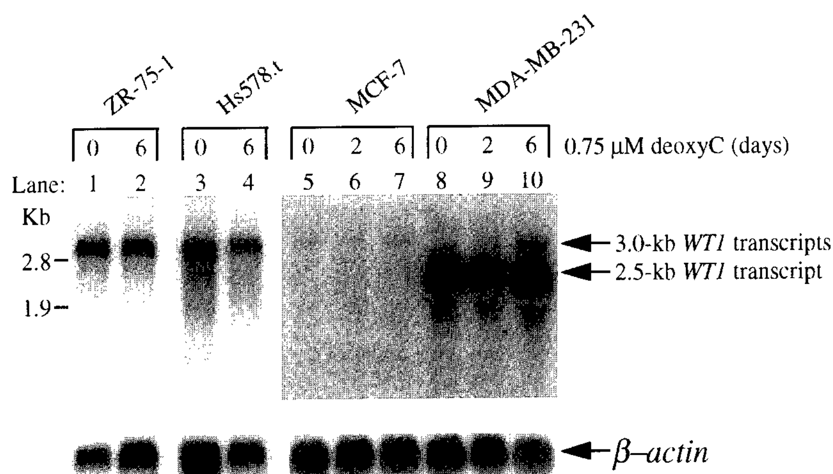

Figure 3. Northern hybridization analysis of *WT1* gene expression in breast cancer cells. ZR-75-1, Hs578t, MCF-7, and MDA-MB-231 cells were either untreated or treated with 0.75 μM 5-aza-2′-deoxycytidine (deoxyC) for two or six days as indicated. Total RNA (20 μg) from these cells was subjected to northern hybridization analysis with a cDNA probe, WT1–7, containing *WT1* exons 7–10 (481 bp). The filter was reprobed with *β-actin* cDNA (1.1 kb). Representative molecular weight markers (from the RNA MW I ladder, Boehringer Mannheim) are shown at left.

ler molecular weight was identified at a high level in MDA-MB-231 cells, and its expression was not altered by deoxyC treatment. Based on a shorter autoradiographic exposure, the message was estimated to be 2.5-kb. All samples were assayed for the *β-actin* mRNA level to enable a relative comparison of loaded RNA amounts (Figure 3, lower panel).

Characterization of the 2.5-kb transcript in MDA-MB-231 cells

RT-PCR analysis aided in identifying the possible composition of the novel 2.5-kb transcript in MDA-MB-231 cells (Figure 4). Amplification product derived from *WT1* exon 1 was not detected in the untreated cells, but was observed in the cells treated with deoxyC (upper panel). The amplified product spanning exons 7 and 8, however, was present in the untreated cells and at a greater level in the treated cells (lower panel). Both cDNA products of exon 1 and exons 7 and 8 were equally amplified in a control sample. The fact that RT-PCR failed to amplify an exon 1 product but amplified cDNA across exons 7 and 8 in the untreated MDA-MB-231 cells indicated that the 2.5-kb transcript described above may not contain the *WT1* first exon. As further evidence of this fact, the first exon has been reported as 454 bp [26], and our Northern analysis demonstrated that this transcript was ~500 bp shorter than the normal 3.0-kb transcripts. The amplified product detected in the treated cells using the *WT1* exon 1 primers represented the 3.0-kb transcripts restored by the demethylation treatment. This observation is consistent with the result that a greater level of cDNA products from exons 7 and 8 was present in the treated cells than in the untreated cells due to the re-expression of the 3.0-kb transcripts.

To determine whether a gene rearrangement or chromosomal translocation involving the *WT1* 5′ end was responsible for expression of the 2.5-kb transcript, restriction mapping analysis of the *WT1* promoter and first exon regions was performed (Figure 5). The known restriction sites of the three endonucleases used in the Southern hybridization (*pvu* II, *Taq* I, and *Xho* I) are indicated in Figure 1A. The results show that the restriction patterns of both MDA-MB-231 cells and normal breast tissue were the same, suggesting that no major structural alterations of the promoter and first exon regions have occurred in MDA-MB-231 cells.

Discussion

The present study demonstrated that the *WT1* CpG island was hypermethylated in MCF-7 and MDA-MB-231 breast cancer cells. Treatment of these cells with a demethylating agent, deoxyC, partially restored *WT1* expression, providing an association between CpG island hypermethylation and *WT1* gene silencing in the cell lines. We have further shown *in vivo* that the *WT1* CpG island was hypermethylated in 25% (five out of 20 patients) of the examined breast tumors. Thus,

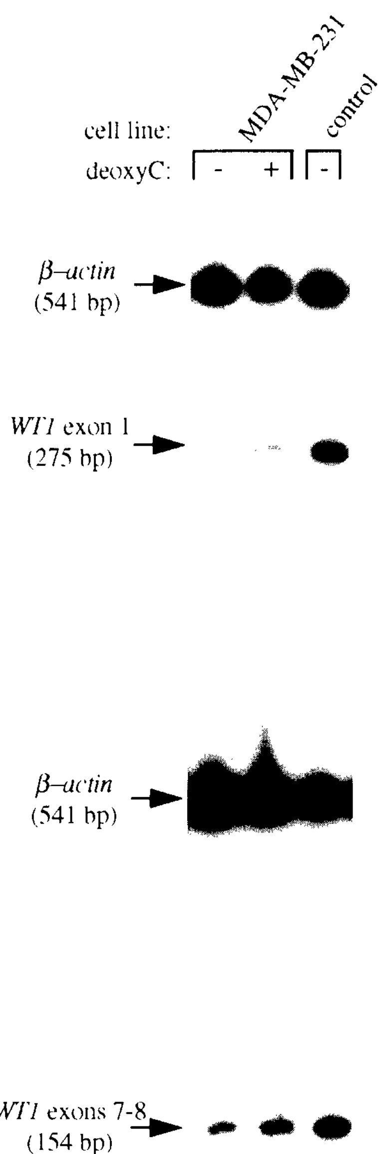

Figure 4. RT-PCR analysis of *WT1* gene expression in MDA-MB-231 cells. cDNA was reverse-transcribed from 4 µg total RNA using oligo(dT) primers and the Superscript Preamplification System (Life Technologies). Diplex PCR amplification using primer sets either for *WT1* exon 1 and *β-actin* (upper panel) or for *WT1* exons 7 and 8 and *β-actin* (lower panel) was performed as described in the text.

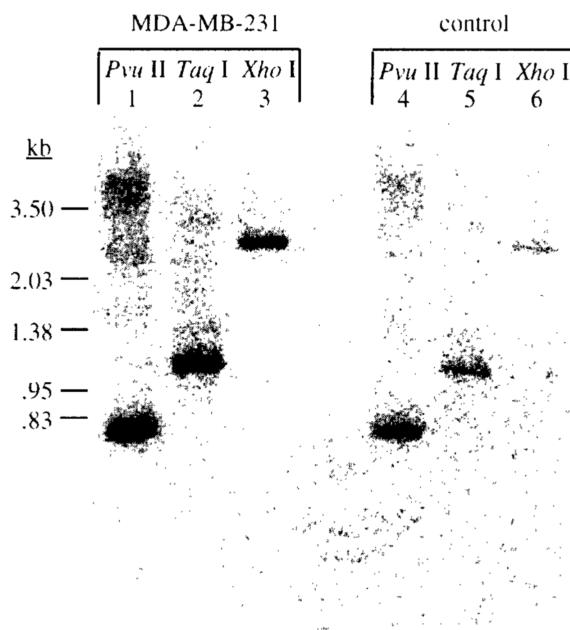

Figure 5. Physical mapping of the *WT1* 5′ region including the promoter and first exon in MDA-MB-231 cells by southern hybridization. Genomic DNA (5 µg) from both MDA-MB-231 cells and a normal breast tissue control sample was separately digested with *Pvu* II(P), *Taq* I (T), and *Xho* I (X), which have known recognition sites within the region of the *WT1* promoter and first exon (see Figure 1a). Hybridization was performed using the WT1–6 probe. Molecular weight markers (MW III, Boehringer Mannheim) are shown at left.

this study adds the *WT1* gene to a growing list of tumor suppressor genes associated with transcriptional down-regulation by DNA methylation [5–9].

Our data suggest that the novel 2.5-kb transcript identified in MDA-MB-231 cells is a *WT1* mRNA variant and that its expression is insensitive to the methylation state of the *WT1* gene (Figures 3 and 4). This shorter transcript may not contain exon 1, and expression of this novel transcript appears not to be influenced by the hypermethylation state of the promoter and first exon regions in the *WT1* gene. Future investigations will focus on cloning and verifying the cDNA sequence derived from this transcript, as well as on characterizing any altered functions of the resulting truncated protein in breast cancer.

Recent evidence indicates a developmental role for *WT 1* in the normal breast. Using immunohistochemical methods, Silberstein et al. [22] reported that *WT1* protein was detectable in mature normal mammary ductal and lobular epithelia, with the protein most abundant in less-differentiated putative stem cells, suggesting that *WT1* plays a differential regulatory role in the development of these mammary structures. Functionally, WTl can negatively regulate the expression of the insulin-like growth factor I receptor, insulin-like growth factor II [27–28] and the transforming growth factor *β* genes [29], all of

which are important in normal breast development. *WT1* inactivation in breast cells could result in an overexpression of these genes, thereby promoting tumorigenic processes. Indeed, Silberstein et al. [22] further observed absent or greatly reduced levels of WTl protein in 68% of a set of 21 primary breast tumors. While the contributions of *WT1* gene deletions, mutations, and antisense suppression [30] to the loss of *WT1* function remain undetermined, our results suggest that hypermethylation of the *WT1* CpG island can be an effective mechanism in attenuating *WT1* expression in breast cancer.

Although this study has focused on breast cancer, suppression of *WT1* expression through hypermethylation may play a role in the development of Wilms' tumor and other forms of human neoplasia. Pulsed-field gel electrophoresis has been used to identify aberrantly methylated sequences within a 300-kb chromosomal region containing the *WT1* gene in Wilms' tumors [31]. More recently, hypermethylation of the *WT1* gene promoter was found in primary colonic adenomas and carcinomas [32]. As *WT1* is expressed in a variety of normal tissues, hypermethylation may provide a common mechanism for modulating *WT1* in a number of different cancer types.

In summary, we have shown that hypermethylation of the *WT1* CpG island was associated with silencing of full length (or normal) *WT1* mRNA expression in MCF-7 and MDA-MB-231 breast cancer cells. Future studies will focus on correlating *WT1* CpG island methylation with possibly altered *WT1* gene expression in primary breast tumors. Moreover, we will study the relevance of the novel 2.5-kb *WT1* transcript to breast cancer progression.

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